Targeted delivery of oligodeoxynucleotides to parenchymal liver cells *in vivo*

Erik A. L. BIESSEN[1], Helene VIETSCH, Erik T. RUMP, Kees FLUITER, Johan KUIPER, Martin K. BIJSTERBOSCH and Theo J. C. VAN BERKEL

Division of Biopharmaceutics, LACDR, Leiden University, P. O. Box 9503, 2300 RA Leiden, The Netherlands

Anti-sense oligodeoxynucleotides (ODNs) hold great promise for correcting the biosynthesis of clinically relevant proteins. The potential of ODNs for modulating liver-specific genes might be increased by preventing untimely elimination and by improving the local bioavailability of ODNs in the target tissue. In the present study we have assessed whether the local ODN concentration can be enhanced by the targeted delivery of ODNs through conjugation to a ligand for the parenchymal liver cell-specific asialoglycoprotein receptor. A capped ODN (miscellaneous 20-mer sequence) was derivatized with a ligand with high affinity for this receptor, N^2-[N^2-(N^2,N^6-bis{*N*-[*p*-(β-D-galactopyranosyloxy)anilino]thiocarbamyl}-L-lysyl)-N^6-(*N*-{*p*-[β-D-galactopyranosyloxy]anilino}thiocarbamyl)-L-lysyl]-N^6-[*N*-(*p*-{β-D-galactopyranosyloxy}anilino)thiocarbamyl]-L-lysine (L_3G_4) (K_d 6.5±0.2 nM, mean±S.D.). Both the uptake studies *in vitro* and the confocal laser scan microscopy studies demonstrated that L_3G_4-ODN was far more efficiently bound to and taken up by parenchymal liver cells than underivatized ODN. Studies *in vivo* in rats showed that hepatic uptake could be greatly enhanced from 19±1% to 77±6% of the injected dose after glycoconjugation. Importantly, specific ODN accumulation of ODN into parenchymal liver cells was improved almost 60-fold after derivatization with L_3G_4, and could be attributed to the asialoglycoprotein receptor. In conclusion, the scavenger receptor-mediated elimination pathway for miscellaneous ODN sequences can be circumvented by direct conjugation to a synthetic tag for the asialoglycoprotein receptor. In this manner a crucial requisite is met towards the application of ODNs *in vivo* to modulate the biosynthesis of parenchymal liver cell-specific genes such as those for apolipoprotein (a), cholesterol ester transfer protein and viral proteins.

Key words: apolipoprotein(a), asialoglycoprotein receptor, cholesterol ester transfer protein, hepatitis B virus, hepatitis C virus, targeting.

INTRODUCTION

Oligodeoxynucleotides (ODNs) have been shown to inhibit gene expression at various levels both *in vitro* and *in vivo* [1–5]. *In vivo*, the efficacy of ODN-induced regulation of genes in specific cell types might be suboptimal owing to the poor accumulation of ODNs in these cells. In addition, untimely elimination of ODNs via renal clearance, degradation and scavenger receptor-mediated uptake [6] might further impair their therapeutic activity. These hurdles can be overcome at least partly by targeted delivery of the ODNs to the desired site of action. A number of approaches have been suggested to facilitate the entry of polyanionic ODNs into the aimed target cell [7–12]. Neutral and cationic liposomes are considered to be attractive ODN carriers because they markedly enhance cellular uptake under conditions *in vitro*. Like native ODNs, however [13], liposomally formulated ODNs are captured mainly by cells of the reticuloendothelial system in lungs, spleen and liver [13–15], as a result of which the ODN concentration in the target cell is suboptimal. After local delivery of ODNs encapsulated in virus capsid-coated liposomes, Morishita et al. [16] enhanced ODN uptake by vascular endothelial cells leading to cell-specific anti-sense effects. Nevertheless, this approach is not feasible for the specific delivery of ODNs to most other cell types such as the parenchymal liver cell (PC).

The PC is the target for anti-sense-mediated down-regulation of the expression of a number of clinically relevant target genes, including that of the atherogenic apolipoprotein(a) [apo(a)] [17], cholesterol ester transfer protein [18,19] and viral proteins from the hepatitis B and hepatitis C viruses [20–22]. A promising way of enhancing the local bioavailability of ODNs in this cell type involves the conjugation of the ODNs to a ligand for a receptor uniquely expressed by PCs, such as the asialoglycoprotein receptor [23]. *In vitro*, glycotargeted delivery to this receptor has been shown after non-covalent complexation of the ODNs to a conjugate of poly-(L-lysine) and asialo-orosomucoid [24–27], whereas a study by Lu et al. [24] also indicated an altered biodistribution of glycotargeted ODNs *in vivo*. In comparison with the rather bulky ODN carriers based on poly-(L-lysine)-asialo-orosomucoid conjugates, the covalent attachment of ODNs to a low-molecular-mass ligand for a cell-specific receptor has the advantage that it is synthetically more accessible, less laborious and pharmaceutically applicable. Hangeland et al. [28] have reported the enhanced uptake of a 7-mer heptathymidinylate

Abbreviations used: apo(a), apolipoprotein(a); ASOR, asialo-orosomucoid; DMEM, Dulbecco's modified Eagle's medium; EC, endothelial liver cell; KC, Kupffer cell; L_3G_4, N^2-[N^2-(N^2,N^6-bis{*N*-[*p*-(β-D-galactopyranosyloxy)anilino]thiocarbamyl}-L-lysyl)-N^6-(*N*-{*p*-[β-D-galactopyranosyloxy]anilino}thiocarbamyl)-L-lysyl]-N^6-[*N*-(*p*-{β-D-galactopyranosyloxy}anilino)thiocarbamyl]-L-lysine; ODN, oligodeoxynucleotide; ODN-AS3′, 5′-CTG.ATT.TCA.G-3′; ODN-AS5′, 5′-NH_2-T.CAG.GTG.CTG-3′; ODN-SE, 5′-TTT.CTG.AAA.TCA.GCA.GCA.CCT.GAG-3′ (nt 82–105 of the Apo-A gene); PC, parenchymal liver cell; Rho, rhodamine.

[1] To whom correspondence should be addressed (e-mail biessen@lacdr.leidenuniv.nl).

(T_7) methylphosphonate by HepG2 cells after conjugation to a GalNAc-terminated glycopeptide. Subsequent studies of this glycoconjugate *in vivo* in mice also showed that hepatic uptake of this conjugate was increased [29]. As short (and in particular uncharged) oligothymidinylates are very poor substrates for hepatic scavenger receptors, which are responsible for the rapid elimination of ODNs by cells of the reticuloendothelial system, the latter results leave unanswered whether longer, charged and miscellaneous ODN sequences can also be redirected to the target cell *in vivo* [6,30]. In this respect it is crucial to analyse the tissue distribution and to identify the cellular uptake sites within the liver by using full-length anti-sense sequences. In the present study evidence is provided from experiments *in vivo* that the untimely elimination of a miscellaneous 20-mer ODN by the above scavenger pathways can be circumvented and, concomitantly, its accumulation by PCs can be enhanced after derivatization with a small-sized synthetic galactoside with high affinity for the asialoglycoprotein receptor.

MATERIALS AND METHODS

Materials

5′-Aminothymidinyl-derivatized phosphodiester ODN (5′-NH_2-T.CAG.GTG.CTG-3′, ODN-AS5′) and a 3′-capped phosphodiester ODN (5′-CTG.ATT.TCA.G-cap, ODN-AS3′; cap is 3′-amino-2-hydroxypropyl) were synthesized at the Department of Organic Chemistry (Professor J. H. Van Boom, Leiden University, Leiden, The Netherlands). The complementary strand ODN-SE (5′-TTT.CTG.AAA.TCA.GCA.GCA.CCT.GAG-3′, nt 82–105 of the *apo*(*a*) gene) [31] and ODN-AS3′-Rho (5′-CTG.ATT.TCA.G-rhodamine) were purchased from Eurogentec (Seraing, Belgium). Human orosomucoid was isolated, subsequently desialylated enzymically, and radioiodinated to yield ^{125}I-labelled asialoorosomucoid (ASOR) as described previously [32].

Synthesis of N^2-[N^2-(N^2,N^6-bis{*N*-[*p*-(β-D-galactopyranosyloxy)-anilino]thiocarbamyl}-L-lysyl)-N^6-(*N*-{*p*-[β-D-galactopyranosyloxy]-anilino}thiocarbamyl)-L-lysyl]-N^6-[*N*-(*p*-{β-D-galactopyranosyloxy}-anilino)thiocarbamyl]-L-lysine (L_3G_4)

p-Aminophenyl β-D-galactopyranose was converted into the phenyl isothiocyanate derivative as follows. A mixture of *p*-aminophenyl β-D-galactopyranose (244 mg, 0.9 mmol) and thiophosgene (0.52 ml, 5.1 mmol) in ethanol/water (4:1, v/v; 50 ml) was stirred for 2 h at room temperature. The excess of thiophosgene was removed by bubbling with N_2 for 1 h at room temperature. After concentration of the solution under reduced pressure, the residue was dissolved in a small volume of distilled water (1–2 ml), and NaOH (1.0 M) was added to bring the pH to 6.0. The solution was concentrated and the residue was chromatographed on a Kieselgel 60 column (40 ml) with dichloromethane/methanol (4:1, v/v) as eluent. Fractions containing *p*-(β-D-galactopyranosyloxy)phenyl isothiocyanate were pooled and freeze-dried to yield 288 mg of a white crystalline powder (0.916 mmol; 102%); R_F 0.74 (dichloromethane/methanol 4:1, v/v); mass 313.2 (calculated: 313.32); ^{13}C-NMR 163.7 (C1-phenyl), 127.8 (C2,6-phenyl), 120.0 (C4-phenyl), 118.9 (C3,5-phenyl), 102.7 (C1-gal, β-configuration), 76.9 (C5-gal), 74.6 (C3-gal), 72.1 (C2-gal), 70.0 (C4-gal), 62.3 (C6-gal).

The activated glycoside (0.10 mmol; 32 mg) and lysyl-lysyl-lysine tetra-acetate (0.03 mmol; 20 mg) were dissolved in a mixture of 0.1 M $NaHCO_3$ (pH 8.5) and dimethylformamide (1:1, v/v; 4 ml); the solution was stirred for 18 h in the dark at room temperature. The progress of the reaction was monitored by TLC. The reaction mixture was concentrated under vacuum; residue was chromatographed on Kieselgel 60 with acetonitrile/water (4:1, v/v) as eluent. Fractions containing product (L_3G_4) were pooled and freeze-dried to yield a whitish powder. R_F (methanol) 0.11; yield 20.0 μmol (34 mg, 67%); mass ($M+Na^+$) 1678.82 (calculated M^+ 1655.82); ^{1}H-NMR (^{2}water/$C^2H_3O^2H$) δ 1.26 [q, 6H, CH_2-γ], 1.57 [q, br, 6H, CH_2-δ], 1.90 [q, 6H, CH_2-β], 3.31 [t, br, 6H, CH_2-ε], 3.68-4.26 [m, 23H, gal H-1 to H-5 and CH-α], 3.97 [d, br, gal H-1(α)]; 5.52 [dd, 8H, gal H-6 and H-6′], 7.05 [m, 16H, phenyl-CH].

Conjugation of ODN-AS5′ with L_3G_4

ODN-AS5′ was conjugated with L_3G_4 as follows. First, a lithium salt of ODN-AS5′ was prepared by precipitation with 5 vol. of 3% (w/v) $LiClO_4$ in acetone. The pellet was freeze-dried, dissolved in water (100 μl), precipitated again with 5 vol. of acetone and freeze-dried. L_3G_4 (2 μmol, 3.3 mg) was dissolved in 75 μl of dimethylformamide/water (2:1, v/v) and 0.5 μl of diisopropylethylamine (2 μmol); 0.79 mg of *O*-benzotriazol-1-yl-tetramethyluronium hexafluorophosphate (2 μmol) and 0.27 mg of 1-hydroxybenzotriazole (2 μmol) were added. The reaction was started by the addition of 70 μg (20 nmol) of ODN-AS5′ in 25 μl of water; the mixture was incubated for 18 h at 37 °C. Then 5 vol. of 3% (w/v) $LiClO_4$ in acetone was added to precipitate the ODN, the pellet was freeze-dried, redissolved in water and precipitated again with 5 vol. of acetone. After freeze-drying, the conjugate was purified by gel electrophoresis in 19% (w/v) polyacrylamide under denaturing conditions in TBE [90 mM Tris/borate/0.1 mM EDTA (pH 8.4)] containing 7 M urea. The putative product was isolated from the gel after detection by UV shadowing (relative electrophoretic mobility, R_F, 1.02; R_F of underivatized ODN-AS5′ 0.40; R_F values of Xylene Cyanol and Bromophenol Blue 1.0 and 0.0 respectively) and desalted on a Sephadex G-25 column, yielding 29 μg of L_3G_4-ODN-AS5′ (31%; 7.26×10^7 $A^{1cm}_{260}\cdot mol^{-1}\cdot cm^{-1}$). Mass (electrospray), 4760.8 (calculated 4760.0). The chemical structures of L_3G_4 and L_3G_4-ODN are shown in Figure 1.

Preparation of [^{32}P]ODN and L_3G_4-[^{32}P]ODN

Internally labelled [^{32}P]ODN and L_3G_4-[^{32}P]ODN (targeting nt 85–104 of the *apo*(*a*) gene) was prepared enzymically from L_3G_4-ODN-AS5′ and ODN-AS3′. ODN-AS3′ was 5′-end phosphorylated with [γ-^{32}P]ATP and T4-polynucleotide kinase by the method of Sambrook et al. [33]. The phosphorylated product was then ligated for 18 h at 8 °C to ODN-AS5′ or L_3G_4-ODN-AS5′ by using T4 DNA ligase in the presence of ODN-SE. The ligation products were isolated by gel electrophoresis on 19% (w/v) polyacrylamide in TBE; the gel was autoradiographed and the ligation product was excised from the gel (R_F 0.88 for [^{32}P]ODN and 1.23 for L_3G_4-[^{32}P]ODN), and subsequently desalted over a Sephadex G25-fine column. The ligation yields of [^{32}P]ODN and L_3G_4-[^{32}P]ODN were approx. 50%. The specific radioactivities of [^{32}P]ODN and L_3G_4-[^{32}P]ODN were typically 3.5–8 Ci/mmol.

Preparation of Rho-conjugated ODN and L_3G_4-ODN

After phosphorylation of the Rho-labelled 3′-end fragment of ODN (ODN-AS3′-Rho) using T4 polynucleotide kinase, ATP, ODN-AS5′ or L_3G_4-ODN-AS5′ were ligated enzymically for 18 h at 8 °C to the phosphorylated 3′-end fragment with T4-polynucleotide ligase. After ligation, the products (clearly visible by the pink staining; R_F 0.96 and 1.30 respectively) were purified by gel electrophoresis on 9% (w/v) polyacrylamide, excised,

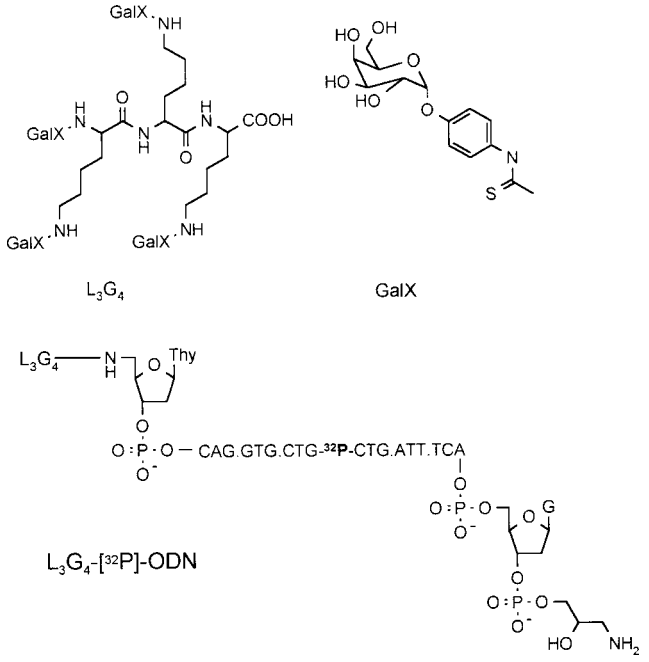

Figure 1 Chemical structures of L_3G_4, X-Gal (GalX) and L_3G_4-[^{32}P]ODN

eluted from the gel and subsequently desalted over Sephadex G-25. The ligation yields, as judged by A_{550}, were 51 % for ODN-Rho and 55 % for L_3G_4-ODN-Rho. The purity of the isolated product as determined by scanning of the relative intensity of the L_3G_4-ODN-Rho band was more than 96 %.

Stability of L_3G_4-[^{32}P]ODN and [^{32}P]ODN in the presence of serum, PCs and titrosomes

To determine the stability of the ODN derivative in serum, internally labelled [^{32}P]ODN or L_3G_4-[^{32}P]ODN (63 pmol, 0.5 μg) was incubated for 180 min with 300 μl of freshly isolated rat serum (diluted 1:1 with PBS, pH7.2, at 37 °C) or with PCs [2×10^6 cells/ml in Dulbecco's modified Eagle's medium (DMEM)+2 % (w/v) BSA, at 4 °C]. As a measure of the stability of L_3G_4-[^{32}P]ODN during lysosomal processing, the ODN (63 pmol, 0.5 μg in 30 μl of water) was incubated for 180 min in sodium acetate buffer (0.1 M, pH 4.5) in the presence of 150 μl of titrosomes (a highly purified preparation of rat liver lysosomes with a specific acid phosphatase activity 25-fold that of liver homogenate prepared by the method of Huisman et al. [34]). At the indicated times, 30 μl samples were taken and ODN was isolated by extraction with phenol and subsequent precipitation in ice-cold propan-2-ol. The pellet was subjected to electrophoresis on denaturing 19 % (w/v) polyacrylamide at an $N/(N-1)$ resolution level [where resolution on the gel was sufficient to discriminate an N-meric from an $(N-1)$-meric ODN] and the gel was autoradiographed on X-OMAT Kodak film and analysed on an Instant-Imager.

Isolation of PCs

Male Wistar rats (approx. 250 g) were anaesthetized by intraperitoneal injection of 20 mg of sodium pentobarbital. PCs were isolated after a 20 min perfusion of the liver with collagenase (type IV, 0.05 %) at 37 °C, as described previously [32]. Viability as judged by the Trypan Blue exclusion assay was greater than 90 %.

Uptake of ^{125}I-ASOR and L_3G_4-[^{32}P]ODN to PCs *in vitro*

For uptake studies of L_3G_4-[^{32}P]ODN, PCs were incubated for 10 min at 37 °C with radiolabelled ligand, put on ice, washed with ice-cold DMEM/2 % (w/v) BSA and incubated for 10 min at 4 °C in a Lab-Shaker (150 rev./min) with DMEM/2 % (w/v) BSA/5 mM EGTA to remove membrane-associated L_3G_4-[^{32}P]ODN. Subsequently, cells were washed once with DMEM/0.2 % BSA and with DMEM; the cell-associated radioactivity was counted and corrected for protein content. Non-specific uptake was determined in the presence of 100 mM GalNAc. To test the effect of various agents that affect lysosomal uptake, cells were preincubated for 30 min at 37 °C with DMEM/2 % (w/v) BSA in the absence or presence of NaN_3 (10 mM), monensin (25 μM), colchicine (100 μM) or sucrose (250 mM), after which L_3G_4-[^{32}P]ODN (10 nM) or ^{125}I-ASOR (10 nM) was added. After incubation for 15 min at 37 °C, the cells were washed twice with washing buffer [50 mM Tris/120 mM NaCl (pH 7.4)] containing 0.2 % BSA and once with washing buffer, after which cell-associated radioactivity was counted and corrected for protein content. During incubation at 37 °C, L_3G_4-[^{32}P]ODN seemed to be stable for 15 min, as judged by analysis by PAGE. Binding of L_3G_4-[^{32}P]ODN to the asialoglycoprotein receptor was studied by incubating isolated rat PCs [(1–1.5) $\times 10^6$ cells] for 2 h at 4 °C in a Lab-Shaker (150 rev./min) with 0–300 nM L_3G_4-[^{32}P]ODN in DMEM/2 % (w/v) BSA. After incubation, cells were washed thoroughly; cell-associated radioactivity was determined and corrected for protein content. The half-life of the ODN glycoconjugate in the presence of PCs at 4 °C was approx. 2 h. The viability of the cells was not affected significantly by the incubation for 2 h at 4 °C.

Confocal laser scan microscopy

Isolated parenchymal rat liver cells [3×10^5 cells in 100 μl of DMEM/2 % (w/v) BSA] were allowed to adhere to a glass layer matrix (25-mm diam.; NO1; Nutacom BV, Netherlands) by incubation for 60 min at 4 °C with DMEM/2 % (w/v) BSA. Subsequently, cells were washed with DMEM/2 % (w/v) BSA; L_3G_4-ODN or ODN (100 nM) was added in the presence or absence of 100 mM lactose and incubated for 20 min at 37 °C. Binding and uptake of fluorescent label by parenchymal cells were detected via confocal laser scan microscopy on a Bio-Rad Slow-scan MRC 500 apparatus equipped with a HeNe triple emitter (543 nm). Image analysis was performed with a Kalman collection filter (ten scans).

Serum clearance and liver association *in vivo*

Male Wistar rats (approx. 250–300 g) were anaesthetized by intraperitoneal injection of 15–20 mg of sodium pentobarbital. [^{32}P]ODN or L_3G_4-[^{32}P]ODN (4 μg in 500 μl of PBS) was injected into the inferior vena cava; at the indicated times the radioactivities in serum and liver were determined as described previously [35]. To assess the involvement of asialoglycoprotein receptors in liver uptake we monitored the liver uptake of L_3G_4-[^{32}P]ODN (4 μg in 500 μl of PBS) injected into the vena cava at 1 min after the injection of GalNAc or GlcNAc (400 mg/kg body weight). To discriminate between extracellular and internalized liver-associated radioactivity of L_3G_4-[^{32}P]ODN, the effect of intravenous injection of GalNAc at 5 or 10 min after the

injection of the radiolabelled ODN on liver uptake was monitored.

Contribution of liver cell types to hepatic uptake

Rats were anaesthetized and injected with [^{32}P]ODN or L_3G_4-[^{32}P]ODN (4 μg in 500 μl of PBS) as described above; 10 min after injection, the vena porta was cannulated and the liver was perfused with collagenase (0.01 %, w/v) (8 °C), and PCs, Kupffer cells (KCs) and endothelial liver cells (ECs) were isolated by centrifugal elutriation as described previously [35]. The purity of PCs was more than 95 % and viability was greater than 90 %; the purities of the EC and KC preparations were more than 85 % and 90 % respectively. The relative contributions of the cell types to the total liver uptake was calculated from the uptake into PCs, ECs and KCs (in ng/mg wet weight), assuming that the PCs, ECs and KCs constituted 92.5 %, 2.5 % and 3.1 % of the total liver weight [35].

Data processing

Saturation binding data and substrate curves of initial uptake were analysed according to a single-site binding model by using non-linear regression (Graph-PAD; ISIS Software, Motulsky). Displacement studies were analysed in accordance with a single-site competitive displacement model by using non-linear regression [36]. The statistical significance of the differences was quantified by Student's *t* test.

RESULTS

Synthesis of L_3G_4-ODN

The tetra-antennary cluster galactoside L_3G_4 (for structure see Figure 1) was synthesized from trilysine and *p*-(β-D-galactopyranosyloxy)phenyl isothiocyanate in 67 % yield. The structural identity of the product was established by NMR and MS. The high affinity of L_3G_4 (see below) allowed further application as targeting device to achieve the specific delivery of anti-sense ODN to hepatocytes. Conjugation of L_3G_4 to the anti-sense ODN was accomplished by a two-step procedure. In the first step, L_3G_4 was linked to the 5′-end part of the anti-sense ODN for apo(a) (ODN-AS5′). In the second step, this ODN was ligated to the phosphorylated and 3′-capped ODN-AS3′ (of the gene for apo(a)). The complete ODN targets a sequence on the apo(a) mRNA (nt 85–104) that is very specific for apo(a) (and not for plasminogen), that is highly accessible (being uninvolved in hairpins or pseudoknots) and that is close to the translation initiation site. Coupling of L_3G_4 to the amino group of the terminal 5′-aminothymidinyl group from ODN-AS5′ was performed in dimethylformamide/water (1:1, v/v) with *O*-benzotriazol-1-yl-tetramethyluronium hexafluorophosphate and 1-hydroxybenzotriazole as cross-linking reagents, and di-isopropylethylamine as catalysing base. L_3G_4-conjugated ODN-AS5′ was produced in 31 % yield and was easily purified from the non-conjugated ODN-AS5′ and free L_3G_4 by electrophoresis on a denaturing 19 % (w/v) polyacrylamide gel. It displayed a slightly lower electrophoretic mobility than the 24-mer ODN-SE (Figure 2, lanes C and F respectively). The product was excised, eluted from the gel and desalted on a Sephadex G25 column. MS results were in agreement with the presumed chemical structure. The coupling efficiency was moderate, despite the 100-fold molar excess of L_3G_4, the coupling reagent and the catalysing base. Attempts to increase the coupling yield by changes in solvent composition or coupling reagents were not successful. The pursued procedure led to markedly higher yields than reactions catalysed by dicyclohexylcarbodi-imide, dicyclohexylcarbodiimide/1-hydroxybenzotriazole, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide or *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline. Furthermore, dimethylformamide/water seemed to be superior to dimethylacetamide/water or acetonitrile/water mixtures or water alone. Efforts to couple L_3G_4 to the 5′-amino-derivatized ODN with the use of a Heinzer base by the method of Oberhauser and Wagner [37] failed for phosphodiester ODNs longer than ten bases, and gave only low yields for smaller ODN sequences. The phosphorylated ODN-AS3′ (Figure 2, lane B) was enzymically ligated to the ligand-conjugated ODN-AS5′ without prior purification. After incubation for 18 h at 8 °C in the presence of the complementary strand (ODN-SE), T4-DNA ligase and ATP, the ligation product was isolated by gel electrophoresis (Figure 2, lane E). The ligation product, L_3G_4-[^{32}P]ODN (Figure 2, lane A) ran at an electrophoretic mobility comparable to that of a 27-mer ODN ($R_F = 0.0555N - 0.2521$; $r^2 = 0.9731$), and considerably more slowly than [^{32}P]ODN-AS3′ (lane B), L_3G_4-ODN-AS5′ (the upper band in lane F) or non-conjugated [^{32}P]ODN (lane D). An extended cooling protocol for annealing L_3G_4-ODN-AS5′ and [^{32}P]ODN-AS3′ to the complementary ODN-SE strand seemed to be critical for obtaining good ligation yields. The presence of the bulky L_3G_4 moiety did not significantly affect ligation yields, suggesting that the glycoside group does not interfere with ODN hybridization to the target sequence.

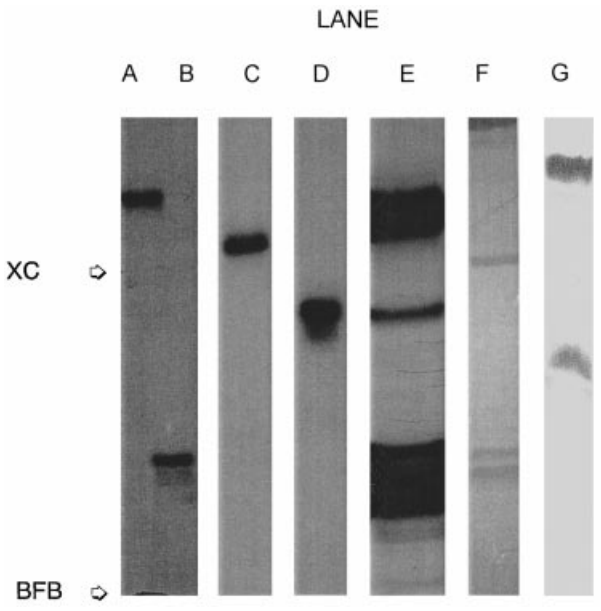

Figure 2 Analysis of the anti-sense ODN derivatives by PAGE [19 % (w/v) gel] under denaturing conditions

Lane A, L_3G_4-[^{32}P]ODN; lane B, [^{32}P]ODN-AS3′, lane C, [^{32}P]ODN-SE; lane D, [^{32}P]ODN; lane E, crude mixture of the ligation of L_3G_4-ODN-AS5′ to [^{32}P]ODN-AS3′ (the upper band is the ligation product L_3G_4-[^{32}P]ODN); lane F, crude mixture of the coupling reaction of L_3G_4 to ODN-AS5′ (UV shadowing; the upper band is L_3G_4-ODN); lane G, crude reaction mixture of the ligation of L_3G_4-ODN-AS5′ to Rho-ODN (Rho scanning at 540 nm; the upper band is L_3G_4-ODN-Rho, whereas the lower band is Rho-ODN). Arrows indicate the positions of the mobility markers Bromophenol Blue (BFB) and Xylene Cyanol (XC).

Binding and uptake studies *in vitro*

We further investigated whether the glycoconjugated ODN was also efficiently and specifically taken up by PCs *in vitro*. Com-

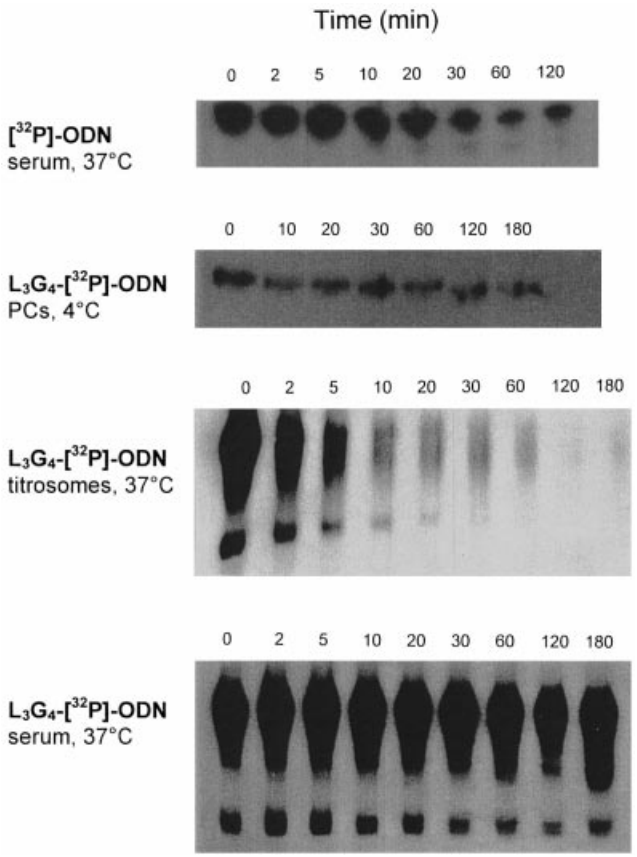

Figure 3 Stability of L_3G_4-[^{32}P]ODN and [^{32}P]ODN in the presence of serum, PCs or titrosomes

L_3G_4-[^{32}P]ODN or [^{32}P]ODN (10 nM) was incubated for 180 min in rat serum (diluted 1:1 with PBS at 37 °C) in the presence of titrosomes (a cellular fraction highly enriched in lysosomal enzymes) (0.1 M sodium acetate buffer, pH 4.5 at 37 °C) or in the presence of PCs [2×10^6/ml in DMEM/2% (w/v) BSA at 4 °C]. At the indicated times, samples (30 μl) were taken and put on ice. The [^{32}P]ODN derivative was isolated by extraction with phenol/chloroform and subsequent precipitation with ice-cold propan-2-ol. The precipitates were subjected to PAGE [19% (w/v) gel] under denaturing conditions (80 mM Tris/90 mM boric acid/2 mM EDTA/7 M urea), and the gels were subsequently analysed with a PhosphorImager. The major bands had electrophoretic mobilities identical to those of untreated [^{32}P]ODN or L_3G_4-[^{32}P]ODN.

petition studies of ^{125}I-ASOR binding *in vitro* to isolated rat hepatocytes showed that free L_3G_4 was able to inhibit the binding of ^{125}I-ASOR to the asialoglycoprotein receptor in a competitive fashion. The affinity was in the low nanomolar range (K_d 6.5 nM, pK_i 8.19 ± 0.01) (results not shown). Subsequently, we tested whether L_3G_4 was also recognized by the asialoglycoprotein receptor after conjugation to the decanucleotide ODN-AS5′. L_3G_4-ODN-AS5′ inhibited the binding of ^{125}I-ASOR with a K_i of 23 nM (pK_i 7.63 ± 0.11). Apparently, derivatization of L_3G_4 with ODN-AS5′ decreased its affinity for the asialoglycoprotein receptor only slightly. In contrast, underivatized ODN-AS5′ was unable to displace the binding of ^{125}I-ASOR at concentrations of up to 200 nM.

To assess the relative contribution of the asialoglycoprotein receptor to the association of L_3G_4-[^{32}P]ODN with hepatocytes, we studied the interaction of internally labelled L_3G_4-[^{32}P]ODN with isolated hepatocytes. First, equilibrium conditions for L_3G_4-[^{32}P]ODN binding at 4 °C were assessed by analysing the kinetics of its association to hepatocytes. In the presence of PCs, L_3G_4-[^{32}P]ODN was stable for 2 h at 4 °C (Figure 3). Equilibrium

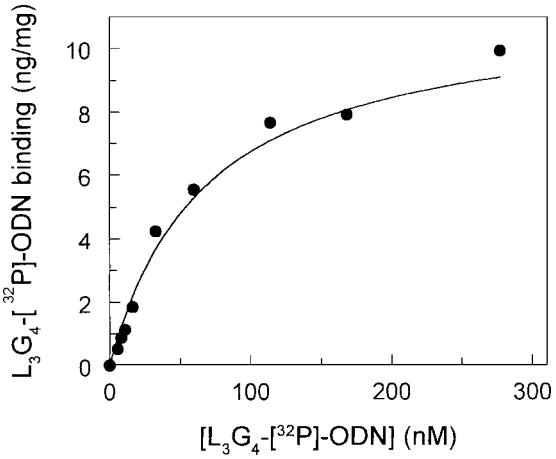

Figure 4 Saturation binding of L_3G_4-[^{32}P]ODN to isolated rat PCs

PCs (10^6 cells per 500 μl) were incubated for 2 h at 4 °C in DMEM/2% (w/v) BSA with 0–270 nM L_3G_4-[^{32}P]ODN in the absence or presence of 100 mM GalNAc. After incubation, cells were put on ice and washed thoroughly; membrane-bound radioactivity was determined and corrected for protein content. The specific binding, defined as the differential binding in the presence and in the absence of GalNAc (mean of a duplicate experiment), is plotted against the ligand concentration.

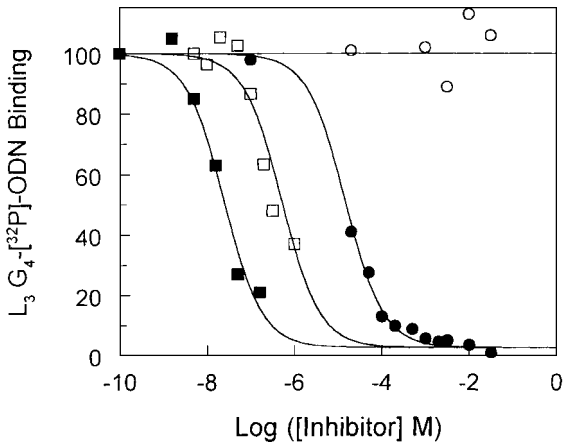

Figure 5 Competition *in vitro* for L_3G_4-[^{32}P]ODN binding to rat PCs by various inhibitors

Rat PCs (10^6 cells per 500 μl) were incubated for 2 h at 4 °C in DMEM/2% (w/v) BSA with ^{125}I-ASOR (10 nM) in the presence of asialo-orosomucoid (■), GalNAc (●), unlabelled L_3G_4-ODN (□) or ODN (○). After incubation, cells were washed thoroughly; cell-bound radioactivity was determined and corrected for protein content. The specific binding, expressed as the percentage of control binding in the absence of inhibitor, is plotted against the log of the inhibitor concentration.

binding at 1 nM L_3G_4-[^{32}P]ODN was achieved within 2 h of incubation (results not shown). Saturation binding studies of L_3G_4-[^{32}P]ODN indicated that binding was monophasic, saturable (B_{max} 11 ± 1 ng/mg) and of high affinity (K_d 68 ± 13 nM) (Figure 4). Subsequently, we investigated the effect of various inhibitors of the asialoglycoprotein receptor on the binding of L_3G_4-[^{32}P]ODN to hepatocytes (Figure 5). ASOR, GalNAc and unlabelled L_3G_4-ODN were each able to inhibit L_3G_4-[^{32}P]ODN binding by 80–90%, whereas GlcNAc was ineffective. From the competition curves, the pK_i values could be calculated, being

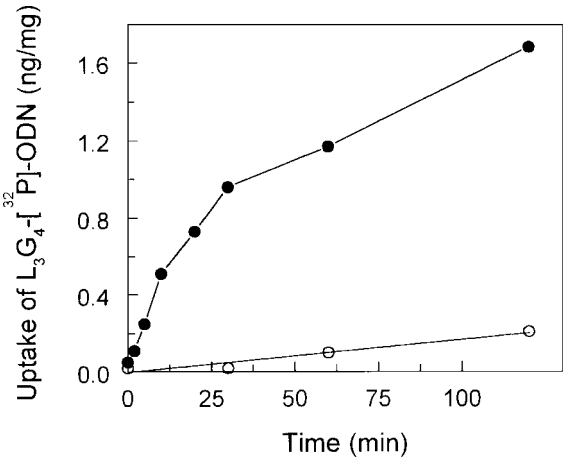

Figure 6 Kinetics of uptake of L_3G_4-[^{32}P]ODN by isolated rat PCs

PCs (10^6 cells per 500 μl) were incubated for 0–2 h at 37 °C in DMEM/2% (w/v) BSA with 10 nM L_3G_4-[^{32}P]ODN in the absence (●) or presence (○) of 100 mM GalNAc. After incubation, cells were put on ice and washed thoroughly; membrane-associated radioactivity was removed by incubation with EGTA (see the Materials and methods section). After a subsequent cell wash, the cell-associated binding (uptake) was determined and corrected for protein content.

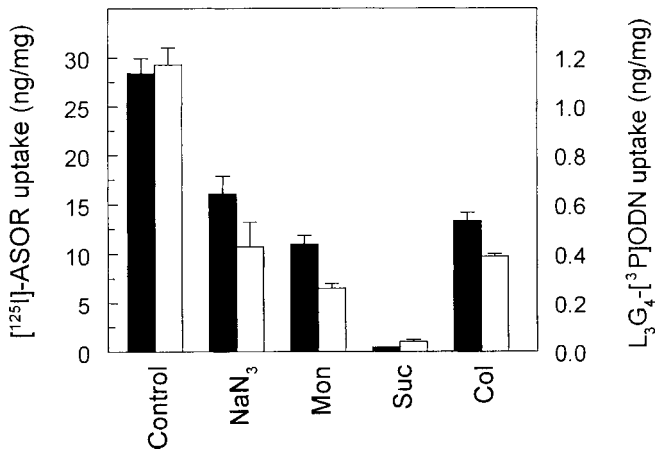

Figure 7 Effect of various uptake inhibitors on the uptake of L_3G_4-[^{32}P]ODN (open bars) and ^{125}I-ASOR (filled bars) by rat PCs

Rat PCs (10^6 cells per 500 μl) were incubated for 30 min at 37 °C with DMEM/2% (w/v) BSA (control) or DMEM/2% (w/v) BSA supplemented with NaN_3 (10 mM), sucrose (Suc, 250 mM), monensin (Mon, 10 μM) or colchicine (Col, 100 μM). Subsequently, L_3G_4-[^{32}P]ODN or ^{125}I-ASOR was added to a final concentrations of 100 nM or 10 nM respectively. After incubation for 15 min at 37 °C, the cells were put on ice, washed once with DMEM/2% (w/v) BSA and incubated for 10 min at 4 °C with EGTA [5 mM in DMEM/2% (w/v) BSA] to remove EGTA-releasable membrane-associated radioactivity. Finally, the cells were washed thoroughly; cell-associated radioactivity was determined and corrected for protein content.

6.59 ± 0.20 (unlabelled L_3G_4-ODN), 7.94 ± 0.10 (asialo-orosomucoid) and 4.88 ± 0.06 (GalNAc).

We then investigated whether these glycoconjugated ODNs were also efficiently and specifically taken up by PCs *in vitro*. The uptake of L_3G_4-[^{32}P]ODN by PCs, i.e. the total cell-associated radioactivity after removal of membrane-bound ligand by treatment with 5 mM EGTA, proceeded linearly with time for 10–15 min and tended to level off after 25 min of incubation (Figure 6). Within the first 20 min, the rate of L_3G_4-[^{32}P]ODN uptake was 35-fold the non-specific uptake in the presence of 100 mM GalNAc. To investigate whether L_3G_4-[^{32}P]ODN uptake involved the classical pathway, we measured the effect of various uptake inhibitors on the internalization of L_3G_4-[^{32}P]ODN by PCs in comparison with that of ^{125}I-ASOR (Figure 7). All of the tested agents decreased the uptake of L_3G_4-[^{32}P]ODN. NaN_3 (10 mM), monensin (0.025 mM) and colchicine (0.1 mM) each inhibited uptake by approx. 70%, whereas sucrose (200 mM) prevented uptake almost completely (approx. 96% inhibition). In agreement, the uptake of ^{125}I-ASOR was similarly decreased after incubation with NaN_3, monensin, colchicine or sucrose.

Confocal laser scanning fluorescence of L_3G_4-ODN-Rho and ODN-Rho

From the above studies *in vitro* it is evident that uptake into PCs is greatly facilitated by the derivatization of ODN with L_3G_4. To reveal the stimulatory effect of glycoconjugation on cell binding and uptake, we analysed the interaction of the Rho-labelled antisense ODNs (ODN-Rho and L_3G_4-ODN-Rho) with rat PCs in real time by confocal laser scan microscopy. L_3G_4-ODN-Rho or ODN-Rho was prepared as described above, in good yield, starting from 3′-Rho-modified ODN-AS3′ (Figure 2, lane F). Competition studies showed that the affinity of the Rho-labelled glycoconjugate for the asialoglycoprotein receptor was close to that of the underivatized compounds (K_i 6.71 ± 0.07, compared with 6.59 ± 0.2 for underivatized L_3G_4-ODN; results not shown). Confocal laser scanning fluorescence analysis of rat PCs was performed after 3 and 15 min of incubation at 37 °C with ODN-Rho (100 nM) or L_3G_4-ODN-Rho (100 nM). No significant fluorescent staining was observed after incubation with ODN-Rho at both time-points. In contrast, PCs incubated with L_3G_4-ODN-Rho showed bright staining at both 3 and at 15 min of incubation (Figure 8). At 3 min, fluorescence was localized mainly in a bright cellular lining, indicative of membrane binding of the fluorescent label. After 15 min of incubation, most of the fluorescence was observed intracellularly, both in spots (indicative of the lysosomal and endosomal compartments) and, in a more diffuse form, in the cytosol. As a measure of the stability of the glycoconjugate in the lysosomal pathway we determined the half-life in the presence of titrosomes (a cellular fraction highly enriched in lysosomal enzymes) (Figure 3). The half-life was 3.1 ± 0.5 min, suggesting that part of the lysosome-entrapped fluorescence at 15 min of incubation reflected degraded ODN. To establish that uptake of the fluorescently labelled ODNs was mediated by the asialoglycoprotein receptor, we also analysed cellular fluorescence after the incubation of PCs with L_3G_4-ODN-Rho in the presence of the inhibitor lactose (100 mM). Fluorescent staining of the cells was fully prevented by excess lactose (Figure 8).

Studies *in vivo*

From the above studies *in vitro* it is clear that uptake by PCs is greatly facilitated by derivatization of ODN with L_3G_4. To confirm that this glycoconjugation also improved the specific accumulation of ODN into PCs *in vivo*, we monitored the uptake by liver and the decay in serum of intravenously injected L_3G_4-[^{32}P]ODN and [^{32}P]ODN in the rat. L_3G_4-conjugated and non-conjugated ODNs were cleared equally rapidly from the bloodstream (Figure 9); within 2 min of injection only 11.5 ± 1.5% and 14.3 ± 1.7% respectively of the injected dose resided in the serum. The hepatic uptake of [^{32}P]ODN amounted to 19.1 ± 0.6%, whereas that of L_3G_4-[^{32}P]ODN was almost quantitative (77 ± 6% of the injected dose). Crucial in interpreting the

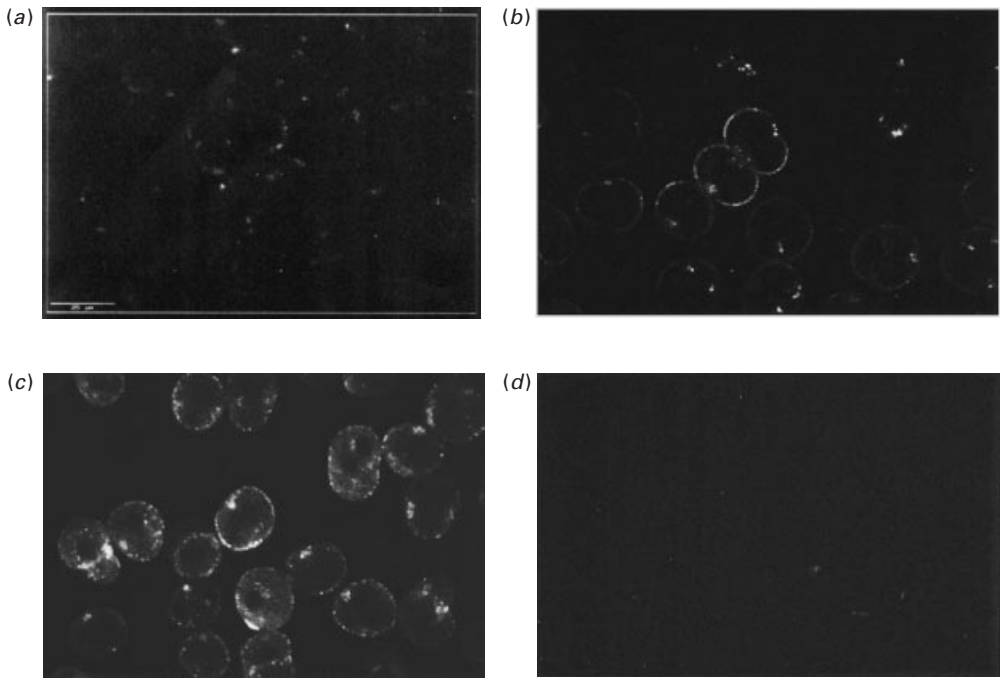

Figure 8 Confocal laser scan microscopy study of the uptake of L_3G_4-ODN-Rho and ODN-Rho by rat PCs

Rat PCs (3×10^5 cells) were adhered to a glass layer matrix by a 15 min incubation at 37 °C. Subsequently, the glass matrices were washed gently, ODN-Rho (**a**) or L_3G_4-ODN-Rho (**b**–**d**) was added to a final concentration of 100 nM [in DMEM/2% (w/v) BSA], and the cells were incubated for 0–15 min at 37 °C in the absence (**a**–**c**) or presence (**d**) of 100 mM β-D-lactose. At 3 min (**b**) and 15 min (**a**, **c**, **d**), cells were confocally analysed for fluorescence. Image analysis was performed with a Kalman filter (ten scans).

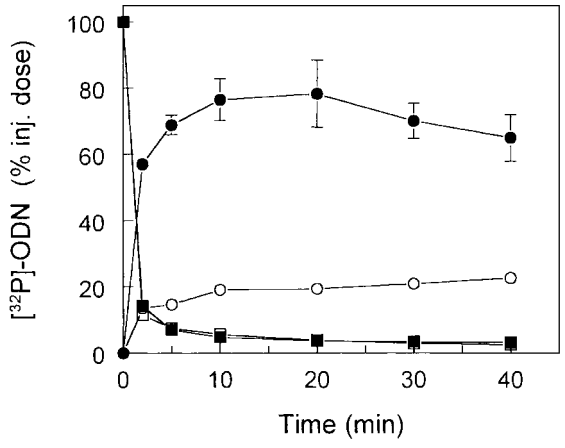

Figure 9 Decay in serum, and uptake by liver, of L_3G_4-[^{32}P]ODN in the rat

L_3G_4-[^{32}P]ODN (●,■) or underivatized [^{32}P]ODN (○,□) (4 μg in 500 μl of PBS) was injected intravenously into rats. At the indicated times, radioactivities in serum (□,■) and associated with liver (○, ●) were determined. Values are means ± S.E.M. for three experiments.

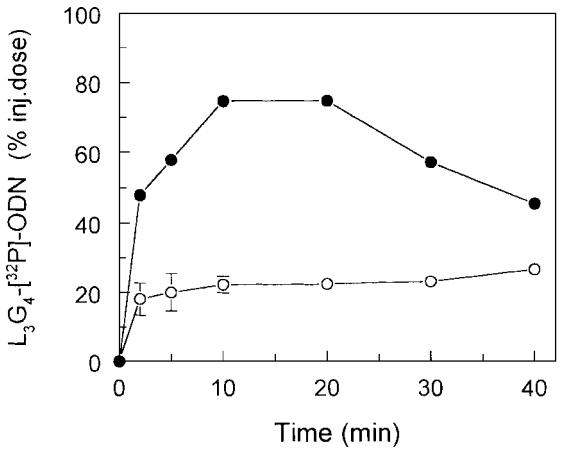

Figure 10 Effect of preinjection of *N*-acetylglycosamines on the uptake of L_3G_4-[^{32}P]ODN by liver

GalNAc (○) or GlcNAc (●) (each 400 mg/kg in 250 μl of PBS) was injected intravenously in rats. At 1 min after injection, L_3G_4-[^{32}P]ODN (4 μg in 500 μl PBS) was administered by intravenous injection into the vena cava. At the indicated times, the liver-associated radioactivities were determined. Values are means ± S.D. of triplicate determinations for GalNAc-treated animals and of duplicates for GlcNAc-treated rats.

above pharmacokinetic data of L_3G_4-[^{32}P]ODN is the stability of L_3G_4-[^{32}P]ODN under conditions *in vivo*. An analysis of the stability of the 3′-capped L_3G_4-[^{32}P]ODN at 37 °C in the presence of serum showed that the glycoconjugate was relatively stable (Figure 3): only 40% of the ODN derivative was degraded during 3 h of incubation. The half-life of the glycoconjugate in serum was 200 ± 20 min, approx. 10-fold that of underivatized [^{32}P]ODN (19 ± 6 min) (results not shown). The induced uptake into liver was prevented almost completely by the preinjection of 400 mg/kg of GalNAc, which blocks galactose-receptor-mediated uptake (Figure 10). The preinjection of GlcNAc, which

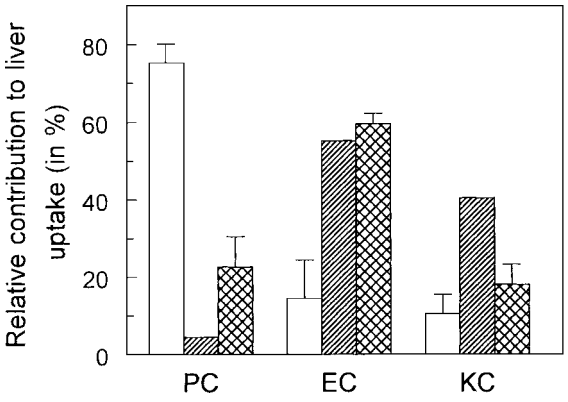

Figure 11 Relative contributions of various cell types to the uptake of L_3G_4-[^{32}P]ODN (open bars) or L_3G_4-[^{32}P]ODN after the preinjection of GalNAc (hatched bars) or [^{32}P]ODN (cross-hatched bars) by liver

[^{32}P]ODN (4 μg in 500 μl of PBS) or L_3G_4-[^{32}P]ODN (4 μg in 500 μl of PBS) was injected into rats 1 min after the preinjection of PBS (250 μl) or GalNAc (150 mg in 250 μl of PBS). PCs, ECs and KCs were isolated from the liver 10 min after injection of the radiolabel; the cellular radioactivity was counted. Values (except those for GalNAc-treated rats) are means $\pm$ S.D. for three experiments and are expressed as percentages of the total uptake by liver.

does not interfere with galactose receptor-mediated substrate recognition, had no effect on the uptake of L_3G_4-[^{32}P]ODN by liver. The underivatized ODN was primarily internalized by ECs (54 %) and KCs (41 %) but not by PCs (5 %). In contrast, the uptake of L_3G_4-[^{32}P]ODN by liver could be mainly attributed to PCs (75$\pm$4 % of the total liver uptake), whereas ECs and KCs contributed only 16$\pm$7 % and 13$\pm$3 % respectively to uptake by liver (Figure 11). The preinjection of GalNAc decreased uptake by PCs by 70 % to 23$\pm$7 %, but increased uptake by KCs and ECs, suggesting that only uptake by PCs was mediated by galactose-recognizing receptors. To verify the nature of the liver-associated ODN, we investigated whether the liver-associated ODN could be released from the liver by displacing the extracellularly bound ODN through the injection of 400 mg/kg GalNAc at 5 and 10 min after the administration of L_3G_4-[^{32}P]ODN. It can be concluded from Figure 12 that liver-associated radioactivity was not significantly affected after GalNAc injection, suggesting that the liver-associated radioactivity reflected internalized, non-releasable L_3G_4-[^{32}P]ODN rather than extracellularly bound L_3G_4-[^{32}P]ODN.

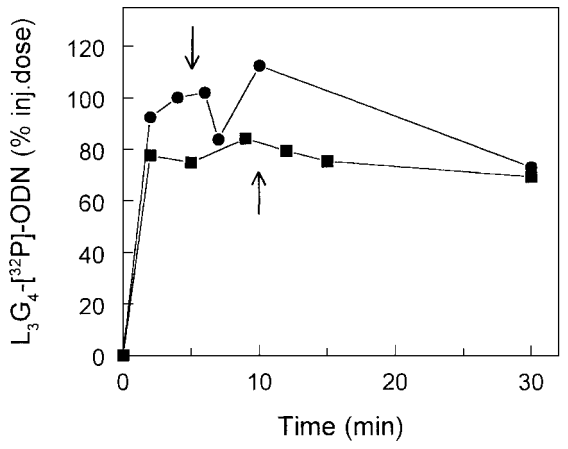

Figure 12 Release of liver-associated L_3G_4-[^{32}P]ODN by intravenous injection of GalNAc in the rat

L_3G_4-[^{32}P]ODN (4 μg in 500 μl of PBS) was injected intravenously into rats. At 5 min (●) and 10 min (■) after injection of the radiolabel (indicated by the respective arrows), GalNAc (400 mg/kg, in 250 μl of PBS) was administered by intravenous injection. At the indicated times, the liver-associated radioactivities were determined. Values are means for two experiments.

DISCUSSION

The application *in vivo* of anti-sense ODNs for the modulation of the expression of target genes in the PC is seriously hampered because it does not accumulate markedly in this cell type. We show in the present study that this drawback can be overcome by glycoconjugating ODNs to a synthetic ligand for the asialoglycoprotein receptor. Previous studies had illustrated that the bioavailability of drugs and genes at the aimed site can be considerably improved through targeting [24,26,29,38–43]. A requisite for successful glycotargeting to the PC is the availibility of a high-affinity ligand for a PC-specific receptor such as the asialoglycoprotein receptor [23]. Various research groups have recently designed glycopeptide mimics of multivalent N-linked oligosaccharides that display nanomolar affinities for the asialoglycoprotein receptor [41–44]. These ligands generally contain glycoside units that are attached to a small peptide scaffold. We have synthesized a tetra-antennary lysine-based galactoside (L_3G_4) with an affinity as great as the aforementioned glycopeptides (K_i 6.5 nM) using an accessible two-step synthetic protocol. Although coupling yields might seem moderate, solution-phase acylation reactions between negatively charged ODNs and carboxylic groups are considered notoriously difficult [28,29,37,44]. Similar yields are reported for solution-phase coupling by Oberhauser and Wagner [37], whereas the two-step procedure used by Hangeland et al. [28] to conjugate a heptathymidine to a triantennary glycopeptide in solution by using cystamine and a thiol/amine cross-linker resulted in only 14 % overall yield.

Competition studies of the binding of ^{125}I-ASOR to hepatocytes established that the affinity of L_3G_4 for the asialoglycoprotein receptor was decreased only slightly after conjugation with ODN-AS5′, suggesting that L_3G_4 might be an appropriate homing device for the targeting of ODNs to the asialoglycoprotein receptor. Therefore we ligated glycoconjugated ODN-AS5′ to the ^{32}P-labelled 3′-end part of ODN, ODN-AS3′, to yield a glycosylated internally labelled 20-mer. Although phosphorothioates are intrinsically more stable than phosphodiesters, we preferred to use capped phosphodiesters because of their lower tendency to bind (serum) proteins [45]. Ligation yields were comparable to those of underivatized ODN fragments. This indicates that the presence of the bulky L_3G_4 moiety did not interfere with hybridization to the complementary sense strand, which is a crucial criterion for the anti-sense activity of ODNs. Next we evaluated whether the recognition, internalization and processing of ODNs by hepatocytes was affected by their conjugation to L_3G_4. L_3G_4-[^{32}P]ODN seemed to bind to hepatocytes in a saturable fashion. The binding of L_3G_4-[^{32}P]ODN could be almost completely (more than 95 %) displaced by unlabelled L_3G_4-ODN and by conventional ligands for the asialoglycoprotein receptor (namely GalNAc and ASOR) but not by GlcNAc. The inhibition constants of GalNAc and ASOR for displacing L_3G_4-[^{32}P]ODN were essentially similar to those reported previously, suggesting that L_3G_4-[^{32}P]ODN binding was mediated almost fully by the asialoglycoprotein receptor. Moreover, after 2 h of incubation the specific uptake of L_3G_4-[^{32}P]ODN was 1.6 ng of L_3G_4-[^{32}P]ODN per mg of cell protein, cor-

responding to an intracellular concentration of 40 nM. This is 4-fold the L_3G_4-[^{32}P]ODN concentration in the medium, confirming that L_3G_4-[^{32}P]ODN uptake proceeds by active transport rather than by a diffusion-mediated process. Studies *in vitro* at 37 °C demonstrated that the asialoglycoprotein receptor not only binds glycoconjugated ODN but also mediates its uptake and processing. The uptake of L_3G_4-[^{32}P]ODN and that of ^{125}I-ASOR were decreased similarly by established inhibitors of lysosomal uptake.

Confocal laser scan microscopy data were in close agreement with the binding experiments *in vitro* in that ODN uptake by PCs was also stimulated strongly after glycoconjugation. The kinetics of endocytosis of the glycoconjugate could be chased in real time, showing that, after binding, intracellular ODN is concentrated successively in the endosomal compartment (just below the membrane surface), in lysosomes (smaller and deeper in the cytosol) and, to a smaller extent, diffusely in the cytosol. The accumulation of fluorescence is strongly impaired in the presence of excess lactose, indicating that the uptake of L_3G_4-ODN-Rho is mediated by the asialoglycoprotein receptors.

In concert with the above results of experiments *in vitro*, *in vivo* the uptake of ODNs by liver seemed to be considerably enhanced to almost 80 % of the injected dose by their coupling to L_3G_4. Uptake by liver is close to values reported for a bi-antennary cluster glycoside with high affinity for the asialoglycoprotein receptor [39] and to that of ASOR itself. Because the degradation of the glycoconjugate in the serum proceeds at a much slower rate than hepatic uptake, because uptake by liver can be inhibited by inhibitors of the asialoglycoprotein receptor, and because the uptake of capped phosphodiester ODNs (or ODN degradation products) by liver has been shown to be marginal [6], we can assume that association of the glycoconjugate with the liver reflects uptake of the intact glycoconjugate. An analysis of the liver cell types revealed that PCs were responsible for the induced uptake by liver. The accumulation of L_3G_4-derivatized ODNs in this cell type after derivatization with L_3G_4 was 60-fold that of underivatized ODNs. Hangeland et al. [29] has reported the enhanced hepatic uptake of an uncharged methylphosphonate, dT_7, conjugated to a tris-galactosylated glycopeptide in the mouse. In agreement, we report here that the elimination of miscellaneous, charged and full-length ODN sequences by scavenger receptors can be prevented, and the uptake of the ODN by liver can be enhanced after derivatization of the ODN with a synthetic glycopeptide tag. Moreover, we demonstrate that uptake is mediated by the aimed target receptor, the asialoglycoprotein receptor, and that it reflects internalized ODN.

In conclusion, glycotargeting of ODN sequences by using cluster galactosides is a very effective way of enhancing the local bioavailability of ODN in the PC *in vivo*. Apparently, the natural tendency of ODNs to be eliminated by the reticuloendothelial system (i.e. macrophages, KCs and ECs) and by cells that express scavenger receptor-type proteins [6,46,47] can be overcome through conjugation to a synthetic low-molecular-mass ligand for the asialoglycoprotein receptor. Importantly, the studies of Sugano and co-workers [18,19] illustrate that an improved hepatocyte-specific delivery protocol for anti-sense ODNs leads to enhanced anti-sense activity *in vivo* at doses that are pharmaceutically feasible. A structural analogue (M_6L_5), containing a terminal mannose group instead of a galactose group, was essentially non-toxic at doses of up to 6 mg/kg, which is 100-fold the doses used in this study [48]. Therefore we expect that the use of the high-affinity tag does not itself invoke toxic side effects. Future studies are currently under way to validate the efficacy of glycotargeted ODN in modulating the expression level of clinically relevant target genes in the PC, such as apo(a) [17], cholesterol ester transfer protein [18,19], viral sequences from hepatitis C virus [20,21], and hepatitis B virus [22] with the use of stable analogues of ODN.

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